ETHANOL FERMENTATION STRAINS

PRESENT AND FUTURE REQUIREMENTS
FOR
BIOMASS TO ETHANOL COMMERCIALIZATION

by

James R. Hettenhaus
Chief Executive Assistance

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TABLE OF CONTENTS

EXECUTIVE SUMMARY

Microorganisms, termed ethanologens, presently convert an inadequate portion of the sugars from biomass to ethanol. Operating temperatures are less than desired and the organism performance can be inhibited by components inherent in the process.

Existing recombinant ethanologens have improved the ethanol yield and productivity significantly over natural occurring strains. Further gains are desired for successful commercialization of lignocellulosic feedstock.

The DOE Office of Fuels Development and NREL wanted an industrial perspective of the improvements needed in these recombinant ethanologens. The findings are intended to assist in guiding short-term development efforts the next two to four years. The results also are intended to better define the long-term requirements for the next generation of ethanologens.

Several colloquies – informal discussion groups -- were held. Participants were primarily from existing industrial producers of ethanol. They represented companies that have high potential for extending their resources to commercialize the lignocellulosic process. Others not able to participate in the colloquies were interviewed. In total, about twenty individuals in positions to influence the future direction participated in the study.

Three recombinant fermentation strains were considered to be candidates for short term improvement by the participants: *rSaccharomyces, rE. coli and rZymomonas*. There is a clear preference for yeast by the existing grain ethanol producers, particularly *rSaccharomyces.* This yeast is widely used, known and fits existing equipment. Other strains may require investment in aseptic processing and more care in operating practices. With *E. coli*, there is the food industry and public mind-set to be overcome, along with the technical and economic challenge it has with *rZymomonas*.

Short term improvements desired are a short list of two items, by priority:

1. Improve yield and productivity, specifically achieve simultaneous utilization of sugars at the present glucose fermentation rate with an overall yield of 90%.
2. Lessen sensitivity to inhibition.

Mid- to long term, existing recombinant strains are expected to be surpassed by an organism not yet identified. Higher operating temperatures are wanted: 50° to 65°C as a 1st step, and then 70°C or greater. Cooling needs are reduced, higher productivity results and hydrolysis benefits when carried out simultaneously as in SSF and SSCF. Other improvements desired by the participants include the following:

- Overall yield above 90%.
- pH should remain low -- 3.5 to 4.0 -- to reduce contamination risk.
- No Inhibition.
- Add value via co-product production.

Possible sources for the next generation were addressed in general terms. It is likely to be GRAS, such as a *thermophilic bacillus, B. sterothermophilus, Clostridium* or steep *Lactobacillus* capable of operating at temperatures too high for yeast or fungi.

The ethanologen development requires actual hydrolyzates for the specific biomass, i.e., straw, and corn stover due to the many process interactions of pretreatment, hydrolysis and fermentation. Some felt an intermediate-scale plant is needed to refine process and organism performance prior to attempting to commercialize the design for a 500 to 1,000 or more ton per day plant. Participants concluded the basic work remains to be done.

1. INTRODUCTION

Commercial ethanol fermentation is almost exclusively accomplished with *Saccharomyces* strains of yeast primarily fermenting glucose. The glucose is readily hydrolyzed from starch in corn and other cereal grains. The starch composition in these grains is between 55 to 70%. Co-products, primarily fiber, oil and protein are sold, reducing the overall process cost.

For biomass—lignocellulosic feedstock— the total content of cellulose and hemicellulose is about the same as the starch in cereal grains, but it is more difficult to process. Table 1 shows the composition for various types of biomass (McMillan, 1994).

Table 1
Biomass Composition, weight %

TYPE	Cellulose	Hemicellulose	Lignin	Other
Agricultural Residues	38	32	17	13
Herbaceous Energy Crops	45	30	15	10
Short Rotation Hardwoods	50	23	22	5

Cellulose hydrolysis produces glucose, which is readily fermented with existing organisms in much the same way as has been done for centuries. Hemicellulose hydrolysis produces both hexose and pentose sugars: mannose, galactose, xylose and arabinose that are not all fermented with existing strains. Other compounds— primarily acetic acid— are also produced during the hydrolysis that can inhibit the ethanol fermentation process. By-products include mostly lignin. When compared to existing grain ethanol by-products they have much less food value and are used as fuel.

2. HEMICELLULOSE IMPORTANCE

Successful fermentation of the hemicellulosic sugars is key for achieving commercial success. Hemicellulose represents 33 to 45% of biomass before processing, and 75% in corn fiber from the corn wet milling process—one-third to three-fourths of the equivalent ethanol available. Table 2 summarizes the cellulose and hemicellulose composition of typical feedstocks judged to be likely choices for commercialization.

Table 2
Typical Cellulose and Hemicellulose in Biomass

TYPE	Cellulose	Hemicellulose	
	%Total	% Total	%Carbohydrates
Corn Stover	39-41	30	43
Switchgrass	36-41	32	45
Serica Lespedeza	34-42	20	34
Short Rotation Hardwoods	39-46	21	33
Corn Fiber	18	53	75

The hemicellulose fraction typically produces a mixture of sugars including xylose, arabinose, galactose and mannose. These are both pentosans: xylose and arabinose, and hexosans: galactose and mannose. The quantities are dependent on the material and also the growing environment and storage history of the material. Table 3 provides the hemicellulose composition of typical feedstocks. Appendix B contains additional details and sources for Tables 2 and 3.

TABLE 3
HEMICELLULOSE COMPOSITION IN BIOMASS
Weight Percent

	Hemicellulose Composition			
Type	Galactan	Xylan	Arabinan	Mannan
Corn Stover	1.0-1.2	19-23	1.8-3.4	0.3-0.7
Switchgrass	1.0-1.1	23-25	3.0-3.4	0.1-0.8
Serica Lespedeza	1.6-1.8	10-14	1.3-1.6	2.0-2.5
Short Rotation Hardwood	0.7-1.2	13-17	0.4-1.1	0.9-1.3
Corn Fiber	3.8	25	18	NR

NR = Not Reported

Studies of component variation due to growing environment and storage between crop years are included in the range given in Table 3. The relatively small differences indicate reasonable feedstock stability (Sanderson, et. al., 1997, Wiselogel et. al., 1996, and Johnson, et. al. 1995).

Xylan represents the major sugar source in all the biomass hemicellulose, from 46% for corn fiber to more than 80% for ag residues, herbaceous crops and hardwoods. Prior to 1980 there was no known way to ferment xylose, the major sugar obtained from hemicellulose, to ethanol.

It is an area of intense research. McMillan (1994) has reviewed the efforts directed towards fermenting these hemicellulose hydrolyzates to ethanol, including recombinant organisms. Du Preez (1994), Jeffries and Kurtzman (1994), Hahn-Hägerdal et. al. (1993) and Schneider (1989) have also examined the major problems to be overcome: low productivity and yields, sensitivity to inhibitors and low ethanol tolerance.

3. PRESENT SITUATION

For commercialization of the biomass route to ethanol, significant gains are needed in performance of these ethanologens, especially fermentation rate and yield of sugars from hemicellulose. Multiple approaches are presently being pursued. Recombinant organisms are available but have not been widely accepted.

4. OBJECTIVE

The objective of this study is to obtain information from industry, better defining commercialization requirements for existing recombinant strains and their next generation. To help guide the continued NREL and DOE support of these efforts, an industrial perspective was desired to assist in setting priorities and focus resources to better accelerate progress.

5. APPROACH

Several colloquies – informal discussion groups -- were held to discuss existing recombinant ethanologens, short term improvement possibilities and the mid to long term prospects for the next generation of these strains.

Participants in the colloquies were primarily from existing industrial producers of ethanol. They represented companies that have high potential for extending their resources to commercialize the lignocellulosic process. Much of the infrastructure is in place and they have substantial capability to fund the venture when the opportunity is more firmly established.

Those not able to participate in the colloquies were interviewed. In total, nineteen individuals in a position to influence the future direction were interviewed. A list of participants is given in Appendix A.

6. ETHANOL FERMENTING ORGANISMS

Microorganisms for ethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment.

6.1. Performance Parameters

Strain performance was discussed in terms of the following parameters:

- Temperature Range
- pH Range
- Alcohol Tolerance
- Growth Rate
- Productivity
- Osmotic Tolerance
- Specificity
- Yield
- Genetic Stability
- Inhibitor Tolerance

<u>Temperature</u>

All the recombinant strains are mesophilic organisms and function best between 30° to 38°C. Operating at greater temperatures is desirable for the following reasons:

- High fermentation temperature increases growth rate and productivity exponentially when the ethanologen can thrive at the higher temperature.
- Plant capital cost is less due to higher productivity per unit volume of fermentor vessel and cooling equipment investment is lowered.
- Operating costs are less since less energy is required to maintain desired fermentation temperature and recover the ethanol.
- Contamination risk is less as fewer organisms exist at high temperatures.
- The enzyme hydrolysis process for saccharification – able to operate up to 55°C – may be combined with fermentation, further reducing capital and glucose inhibition.

Temperature effect on alcohol recovery and inhibition was discussed. Stripping off the ethanol at higher temperatures has little effect on either lower recovery costs or alcohol inhibition. Pressure can have an impact. See Appendix D.

pH Range

Most bacteria grow in the range of pH 6.5 to 7.5. Yeast and fungi tolerate a range of 3.5 to 5.0 pH. The ability to lower pH below 4.0 offers a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants.

Simultaneous saccharification and fermentation will be adversely affected, however, at lower pH. T. reesei based cellulases perform best at about pH 5. Present acid cellulases for the textile industry retain 80% activity at pH 4.0. Still lower pH will denature the protein, dependent on time, temperature and alcohol tolerance.

Alcohol Tolerance

The majority of organisms cannot tolerate ethanol concentrations above 10 to15% (w/v). The protein becomes denatured. Higher temperature lowers the tolerance of the organism. At temperatures above 35°C, current strains lose viability at ethanol concentrations of 10% (w/v).

Growth Rate

The organism growth rate is dependent on many other parameters including nutrients, inhibitors, pH and temperature. A high specific growth rate is desired. Typical values for various organisms are provided below (Blanch et. al., 1997).

Organism and Growth Temperature	Nutrient	Growth Rate (hr^{-1})
E. Coli (37°C)	Glucose	0.8-1.4
Saccharomyces cerevisiae (30°C)	Glucose	0.5-0.6
Klebesiella aerogenes	Glycerol	0.5

Productivity

The product of the cell concentration and the specific ethanol production rate determines the overall volumetric process productivity, expressed in grams per liter-hour. The ethanol productivity of *Saccharomyces* strains in current grain-based ethanol production processes is in the range of 2.5 - 4.5 grams per liter-hour.

Osmotic Tolerance

The semipermeable membrane surrounding the cell must be able to withstand wide osmotic pressure changes in extracellular fluids that impact the relative osmotic pressure difference. If not, the cells may be severely damaged or even killed.
The cells may burst in a hypotonic solution, when the solution becomes more dilute than the intracellular fluid. If hypertonic, the cells will shrink from the osmotic pressure difference. Osmotic pressure limits can be one of the factors that restrict maximum substrate concentration.

Specificity

The measured consumption and production of desired components in relation to the theoretical maximum reaction stoichiometry describes the specificity of the organism. Specificity is closely related to the yield coefficients.

Yield

The amount of product formed per unit of substrate consumed by the organism is a useful way to refer to yields. Yields are expressed on either molar or weight basis. For process cost accounting purposes, weight is more meaningful.

In this case the primary stoichiometric equations for the ethanol production are as follows:

1. Pentosan to Pentose, 1.136 weight gain.

$$n\ C_5H_8O_4 + n\ H_2O \rightarrow n\ C_5H_{10}O_5$$

n 132 MWU	n 18 MWU	n 150 MWU
(1 gram)	(0.136 gram)	(1.136 gram)

2. Hexosan to Hexose, 1.111 weight gain

$$n\ C_6H_{10}O_5 + n\ H_2O \rightarrow n\ C_6H_{12}O_6$$

n 162 MWU	n 18 MWU	n 180 MWU
(1 gram)	(0.111 gram)	(1.111 gram)

3. Pentose and Hexose to Ethanol, 0.511 grams per gram hexose or pentose.

Pentose: $3\ C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2$

3 x 150 MWU	5 x 46 MWU	5 x 44 MWU
(1 gram)	(0.511 gram)	(0.489 gram)

Hexose: $C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

180 MWU	2 x 46 MWU	2 x 44 MWU
(1 gram)	(0.511 gram)	(0.489 gram)

The weight yield of pentose from pentosan – xylan and arabinan – is 1.136 grams pentose per gram pentosan. This number results from 150/132, the ratio of the molecular weight of pentose per molecular weight of anhydropentoses that make up pentosans.

The yield of hexose from glucan, mannan and galactan that are hexans from the cellulose and hemicellulose is 1.111 grams glucose per gram hexosan, the molecular weight ratio of 180/162 for glucose and anhydrohexoses.

The yield of ethanol for fermenting is 0.511 grams per gram of hexose or pentose. The overall theoretical yield for conversion is 0.581 grams ethanol per gram pentosan and 0.568 grams per gram hexosan. . . .just a 2.3% difference.

The conversion of other oligosaccharides, mostly dp2 and dp3 sugars, requires their hydrolysis to either a hexose or pentose, resulting in the same chemical gains.

A reduction in yield below theoretical always occurs since the microorganism requires a portion of the substrate for cell growth and maintenance . For *E. coli* and *S. cerevisiae* these values are approximately 0.054 and 0.018 grams of glucose/g dry cell weight-hour respectively (Roels et. al., 1978).

Genetic Stability

Present yeast strains initially adapt to the substrate and after acclimatization increase alcohol production (Cameron et. al., 1997; Linden et. al., 1992). The physiological basis for these adaptations is unknown.

The engineered organism's ability to remain genetically stable after multiple generations is necessary to insure consistent performance.

Recombinant strains where the modification is made to the chromosome generally exhibit good genetic stability after multiple generations occurring over several months. Organisms modified by introduction of extracellular DNA elsewhere in the cell, such as in the plasmid, are typically more susceptible to genetic instability after days or weeks of operation. This usually results in a decline in performance.

Inhibitor Tolerance

McMillan (1994) grouped the fermentation inhibitors into three classes:

1. Compounds originating in the biomass by hydrolysis.

These include organic acids such as acetic, glucuronic and galacturonic acids from the hemicellulose, and phenolic compounds from the lignin. The most inhibitory of these for both yeast and bacteria is acetic acid. Solubilized lignin is also a factor.

2. Compounds formed by degradation of the products resulting from pretreatment and hydrolysis of the biomass.

Furfural from xylose and HMF from glucose lead this group. It is completed by an assortment of aldehydes, acids and alcohols from lignin, sugar and protein degradation.

3. Compounds from other sources.

Metal ions resulting from equipment corrosion, sulfites, SO_2 and lactic acid introduced with other streams containing nutrients, cleaning solutions and backset.

6.2. Process Compatibility

The ability to use existing production equipment and similar processes reduces cost and time for introduction. The ease of recycling the cell mass is another factor. Unless the cell mass offers a value added component, it is advantageous to recycle as much as possible. Ethanol yield and productivity is increased and fewer nutrients are required for cell growth.

Possible regulatory, health and safety issues also require consideration if a new or different strain is employed. Especially important is the continued sale of animal feeds containing cell mass.

While cell mass makes up about 2% of the process, its disposition has a significant impact on cost with existing grain-to-ethanol processors since it is sold as an ingredient in the animal feed, either as gluten feed or distillers grains. Gluten feed from the wet mill process fluctuate widely in price. At this time the feed is $40 to $60/ton, and distillers dried grains from the dry milling process is $60 to $80 or more per ton.

If the cell mass planned for use in the animal feed is from a GRAS organism (generally regarded as safe), FDA approval is normally straightforward. Any organism is considered GRAS if it is derived from well-known, widely distributed, nonpathogenic strains of yeast cultures such as the genus *Saccharomyces*, or if it belongs to a bacterial or mold species that is well characterized, commonly present in food, has a history of safe use and has never been implicated in foodborne intoxication or disease, e.g. B. lichenformis and subtilis and A. niger, A. oryzae.

If it is not from a GRAS organism, lengthy and costly testing is needed to gain approval for is use in the feed. In addition, more processing may be required. For example, if spore formers such as *Bacillus* or known pathogen relatives such as *E. coli, Klebsiella* or *Candida* are chosen, the processed cell mass would likely require continuous monitoring to insure it is free of these agents before released for other use.

For a 1,000 ton per day plant the difference in burning versus selling the cell mass in animal feed is $140,000 to $420,000 annually. The penalty is based on $20 to $60 per ton price difference for 2% of the product mix. The heating value is taken as $10 to $20 per ton at best, compared to gluten feed and distillers grain, currently at $40 to $80 or more per ton respectively.

But the *actual cost would be much higher*-- $1.5 million for wet mill processors and about $5 million per year for dry millers-- since the cell mass cannot be readily separated from the corn fiber or from the distillers grains. ALL the material in the process stream with the cell mass must be considered. The resulting cost penalty is unacceptable.

For biomass processors using lignocellulosic feedstock, lignin is the primary by-product, Table 1. It has a heating value close to coal and no food value. The relatively small portion of cell mass is not readily separated from the lignin. Its disposition cost has less impact. The cell mass is usually considered an energy source for biogas production or boiler fuel.

7. EXISTING RECOMBINANT ORGANISMS

For existing recombinant strains, participants primarily focused on *rSaccharomyces, rE. coli* and *rZymomonas*. Others mentioned included *rKlebsiella, rPichia stipitis, rB. Stearothrermophilus* and *rKluveromyces.* A number of other naturally occurring organisms that convert pentoses to ethanol were named. These included *P. stipitis, Pachysolen tanophilus* and *Candida shehatae.*

Only the following three are seen as viable candidates for the near term:

1. *rSaccharomyces*
2. *rE. coli*
3. *rZymomonas*

7.1. *rSaccharomyces*

Current production organisms are primarily the natural occurring *Saccharomyces* strains of yeast. They have long been used for brewing and fermentation of distilled products. Today they are almost exclusively used for large scale industrial production. Yeast is viewed as hearty and user friendly. Plant staff is familiar with its use.

These strains usually produce 10 to 15% ethanol (v/v) at pH 3.5 to 4.5 at 32° to 38°C with productivity up to 4.5 grams per liter-hour. They have high specificity for alcohol, along with alcohol tolerance up to 20%(v/v) at 30°C. High osmotic pressure tolerance, up to 38% (w/v) sugar, is tolerated without damage to the yeast.

To better leverage these yeast properties, increasing the sugar and alcohol concentrations is proposed (Thomas et. al., 1996, Ingledew et. al., 1998). Pilot studies show operation can be sustained with ethanol concentrations of 18% (v/v) at 28°C. Higher concentrations usually increase viscosity, another constraint to be considered. Also, pretreatment usually dictates a compromise for broth composition.

Process laboratory support is simple. Some plants do not have a microbiologist on staff, relying on trained operating personnel. Yeast is handled in a casual manner. Neither aseptic conditions nor sterilization of feed streams is required, saving both capital and operating cost. In the event a bacterial infection is detected, the pH is lowered to eliminate the contaminant. The largest risk is other “wild” yeast strains that reduce yield.

Yeast cells are recovered from the process streams and recycled to maintain the desired cell density for high yield and productivity. A portion can be removed, dried and sold as a value-added co-product or as an animal feed additive. Some is almost always retained for use as an inoculum. It has already acclimated to the particular process and purchase of fresh yeast is avoided. Its ready availability can provide a production boost or a quick restart when needed. This improves throughput and reduces investment in the seed reactor train.

The present recombinant strains have long induction times for the metabolic pathways, with a definite preference for glucose, then xylose and arabinose, mannose and lastly balking with galactose. Oligomer utilization was described as unacceptable. The greater the degree of polymerization, the poorer the performance, i.e., $dp_4 <<< dp_3 << dp_2$. For *rSaccharomyces*, attempts to introduce arabinose fermenting capabilities have been unsuccessful. This is an important target for the corn wet milling industry with corn fiber that contains 18% arabinan (Table 3.)

Genetic instability is reported when the introduced genes are plasmid borne. The ability to ferment hexosans decreases with time, requiring periodic additions of fresh yeast. From an environmental perspective, the plasmid approach requires that the plasmid does not migrate into the environment. From a process perspective the instability of plasmids is not desirable.

The genetic instability is avoided for *rSaccharomyces* with modified chromosomes. This form has exhibited stable performance over multiple cycles, exceeding one thousand hours with no diminished fermentation capability.

Yeast fermentation also produces some fusel oil and glycerol along with organic acids. While fusel oil is compatible with fuel ethanol uses, these byproducts complicate downstream processing.

Other disadvantages of yeast include its low temperature tolerance and relative genetic complexity, although the latter concern is diminishing. It requires oxygen, albeit a minimal amount.

7.2. *rE. coli*

rE. coli has high productivity and growth, about two times greater than yeast. It also has broad substrate utilization. The organism is well understood with a known genetic system for metabolic pathway manipulation. Its recombinant forms are widely used in the pharmaceutical industry to produce many human drugs, including insulin.

However, its largest hurdle for most existing ethanol producers is the mind-set of *E. coli* with the public as a pathogen at worst, or associated with fecal matter at best. Most colloquy participants also view *rE. coli* as more susceptible to phage problems and genetic instability.

Other process weaknesses associated with *E. coli* include its neutral pH, 6 – 7, relatively poor ethanol tolerance and temperature sensitivity. At 30°C, it will survive but not grow at 7 to 8% (v/v) ethanol. In contrast, yeast will withstand 18 to 20% (v/v) at the same temperature.

Disposition of the cell mass is a concern for all organisms, but more so with *E. coli.* The cells are difficult to recycle relative to yeast. Adding them to an animal feed is also a larger 'mind-set' obstacle due to its unfavorable reputation. Finally, considerable investment is expected to meet the need for inoculum and aseptic processing since existing yeast growing vessels will not be acceptable. In case of process contamination, the turn around time requirements are also greater relative to yeast.

7.3. *rZymomonas*

*rZymomona*s has high ethanol tolerance, about 11% (v/v) at 32°C and converts arabinose and xylose to ethanol. It is relatively fast growing and has good specificity and productivity.

The pH range is 5 to 6, and its performance in non-aseptic situations is highly questionable. Like *E. coli,* the present *rZymomonas* most likely requires aseptic conditions that cannot be met with existing yeast-designed equipment. Temperature is also limited to 35°C, and the organism is sensitive to substrate starvation.

In the late 1980's a naturally selected strain approved by the FDA as an animal feed ingredient was used in the US for ethanol production. The results were favorable (Millichip, 1989). Typical fermentation conditions were 38°C, 12% to 15% ethanol and

3.6 to 3.8 pH. Conditions were anything but aseptic. After some initial problems with seed inoculum scale up, long term, multi-month performance was demonstrated in the production fermentors up to 150,000 gallons. No trials were made on any hydrolyzates of hemicellulose. At least one licensing offer was made. The parties could not agree on terms. Biocom International later decided to abandon the US market. Additional information is in Appendix C.

Other issues include cell mass handling, its disposition, and relative genome knowledge. The FDA approval, see Appendix C, excludes genetically altered forms. Also, the cells are difficult to recycle but their cell mass is lower than yeast and *E. coli*, so it is a smaller problem. *E. coli* and *Saccharomyces* genetic systems are better documented.

8. PREFERENCE

The unanimous choice for commercialization among the participants from the corn ethanol industry for the short term is ***rSaccharomyces.*** For general biomass conversion ***rE.Coli*** has support due to its wide substrate range. Its cell mass is used as a fuel, and disposition concerns are minor. ***rZymomonas*** offers potential for rapid improvement and is a likely alternative for the existing grain ethanol industry.

Yeast is widely used by the current ethanol producers, well known and fits existing equipment, process and products. Other strains will likely require investment in aseptic processing and more care in operating practices. With *E. coli*, there is the food industry and public mind set to be overcome, along with the technical and economic challenges it has with the present *rZymomonas*.

Cell mass addition to animal feeds requires FDA approval. To obtain approval of non-GRAS organisms is often costly and time consuming, in the range of hundreds of thousands of dollars and years to accomplish. The amount depends on previous work and regulatory precedents. *rYeast* disposition as a food additive is the most direct. *rZymomonas* approval is likely to require less time and resources due to the approval of its naturally selected counterpart. *rE. coli* approval is considered more difficult.

Longer term a more temperature resistant strain must be obtained. There was consensus that the existing recombinant strains, including yeast, will not be suitable for the next generation of ethanologens. A new organism remains to be identified and developed. It is likely to be GRAS, such as a *thermophilic Bacillus, B. sterothermophilus, Clostridium* or steep *Lactobacillus* capable of operating at temperatures too high for yeast or fungi.

They may be anaerobes, eliminating the need of air for growth. Since the use of air now is minimal for yeast, current usage can be viewed as the upper limit. While higher temperature is required, extremophiles are probably not needed since the vapor pressure of ethanol is greater than 1 atm at 80°C.

9. IMPROVEMENTS

Commercialization is dependent on many factors including capital investment required, raw material cost and availability and process operating cost. Enhancements to the ethanologens can contribute significantly.

The improvements desired for the short term – using existing recombinant strains – and for the longer term, examining the requirements for the next generation of ethanologens, were described by the participants.

9.1. Short Term

Improvements for the next two to three years should focus on simultaneous utilization of the sugars at the same production rate as glucose, 2.5 to 4.5 grams per liter-hour with no feedback repression of glucose. The overall yield result should be 90% or greater. Based on the biomass composition in Table 3, recombinant organisms with the capability to co-ferment glucose, xylose and arabinose can meet this requirement.

The yield target may be achieved without fermentation of mannose and galactose, which represent just 2% to 5% of total sugar. Production strains that incorporate a cloned fungal maltase gene to reduce dp2 losses in current grain-based production may have a more significant impact.

As a secondary priority, lessening inhibition by strain improvement, reducing by-product production and other side reactions, i.e. acetic, lactic acids, is recommended. Further enhancements are not expected in the relatively short time frame.

For *rZymomonas* and r*E. coli* to be considered, the pH tolerance needs to be 4.0 pH or less and their cell mass disposition resolved in addition to the above suggestions for utilization rates, yield, and inhibition tolerance.

9.2. Mid- to Long Term

Increased operating temperature is viewed as the most important improvement to target four or more years in the future. The increase will likely occur in two steps: the initial step to 55°C range, 45 - 65°C, and then another increase to 70°C or more.

The first jump to 55°C is considered reasonable using the existing knowledge base. The benefits for higher temperature operation include reduced power input and less cooling and fermentation time. Also, higher productivity for fermentation, in excess of 5 grams per liter-hour is expected.

The design issue of separating hydrolysis for 55°C operation, using a cellulase enzyme not inhibited by glucose is largely resolved by operating at 55°C. Simultaneous Saccharification and Fermentation (SSF or SSCF, C for co-fermentation) can be better implemented at the increased temperature. Capital and operating costs are reduced, along with the contamination risk.

Further increases, to 70°C or more enhances the above and also permits the ethanol to be stripped off as it is produced. However, the participants pointed out we are leaving much of the knowledge base when moving to this temperature level. It is more difficult, requiring new learning.

Yields approaching 95% are expected for sugars: all monomers, dp2 and dp3's, and maybe lignin will be fermented. The organism should utilize acetic acid and lignin as a carbon source for its growth or the production of value added co-products.

Production of other value-added co-products should be pursued. This includes enzyme expression for the biomass hydrolysis and possibly something that can be fractionated by existing distillation with minor additions or readily separated from the large process stream volumes.

Integration of existing equipment with the new process reduces the cost of entry. Therefore pH should be as low a possible, 3.5 pH or less without causing other problems such as gypsum formation and scaling of process piping and equipment. This has the advantage of reducing losses to lactobacilli and acetic acid bacteria. Hydrolysate and other contamination problems show up at higher pH – with one caveat noted: acetic acid pK_a is 4.8 at 30°C (McMillan,1994, p. 424). Its toxicity increases rapidly at lower pH because it is the protonated form of the acid that is inhibitory. It is 90% undissociated at 4.0 pH and 95% at 3.5. At 5.5 pH it is just 15% undissociated.

No inhibition should occur with the new strain. This organism most likely results after several iterations using hydrolyzates formed under the "best" conditions for pretreatment and hydrolysis for a specific feedstock. A combination of properly controlled pretreatment and hydrolysis processing that eliminates most inhibitor formation, along with organism engineering to deal with those inhibitors that do occur is required.

Ready disposition of the cell mass is required. Simply settling the cells from solution is the preferred recovery method. Using flocculating strains is one route. Another is achieving high growth rates that generally result in larger cell formation. Stokes' Law says the settling rate velocity is proportional to the square of the particle diameter, and larger cells increase the prospects for gravity separation.

Other ways to deal with cell mass is centrifugation, filtration and immobilization. Centrifugation is capital intensive and has high maintenance cost. Filtration, using a filter aid, generates another waste stream that is not wanted. Cell immobilization may be considered for pilot testing after process simulation cost studies.

Following the colloquies, a process under development by Agrol Ltd is reported to be operating at 70° - 80° C with thermophilic bacteria converting pentose and hexose sugars from hydrolyzed biomass (Richert and Zimmer, 1998). The operation (20L fermentors and 800g EtOH/hr) includes a 1st stage anaerobic fermentor coupled to an aerobic fermentor via microfilters for cell retention and recycling. It is currently being scaled up to 800 kg/hr. According to an Agrol Ltd spokesman, the ethanologens ferment all sugars at nearly equal rates. Except for coniferous woods, the hydrolyzates have little inhibitory effect (Lucas, 1998).

Another approach for 2nd generation microorganisms is developing the ability to produce sugars and ethanol simultaneously, (Hogsett et al, 1992, Lynd, 1996). The result could be still lower capital and operating costs associated with the cellulase production. Also, potentially faster rates and improved yields are expected. These advantages are because the ATP for cellulase production comes from anaerobic ethanol-yielding metabolism rather than from aerobic metabolism yielding CO_2 and water (van Walsum and Lynd, 1998).

10. PROCESS DEVELOPMENT AND VALIDATION

Access to "real" process streams is needed to determine the production organism's requirements and fully test its performance.

For example, to avoid inhibition of the fermentation, it is best to eliminate any inhibitor formation in the pretreatment and hydrolysis steps. Establishing the conditions which will produce minimal inhibitors for each feedstock, and what the inhibitor composition will be is key for developing a robust ethanologen . . .and a successful process.

Similarly, pretreatment and hydrolysis produce oligomers. By characterizing these sugars, the strain properties for their fermentation can be developed.

The multiple interactions between pretreatment, hydrolysis and fermentation performance requires several stages of scale up for prudent process development and validation. Three one-ton per day biomass pilot plants are now available for operation in North America for biomass process development and validation: NREL, Iogen and TVA. Semi-commercial, intermediate size plants of 40 tons and 80 tons per day feed by Iogen and Agrol Ltd. are expected to be operational in late 1999 or early 2000. More immediate, BC International is presently starting up their full size 20 million gallon per year facility in Jennings, LA.

Using existing corn or other grain ethanol production facilities for trials is another option. For *rYeast* strains, this may be an alternative put forward for consideration. In a small plant the economic disruption may be tolerable. However, to make a plant trial with *rZymomonas* or *rE. col*i would be a special case, most likely isolated from other production facilities, with a higher cost.

11. CONCLUSION

Recombinant strains suitable for commercialization now exist. Improving their performance improves the process economics, increasing commercialization opportunities.

Utilization of existing equipment requires an organism similar to what is used now. A *rSaccharomyces* fits this need best for existing grain ethanol producers, along with *rZymomonas*, especially if it is deemed GRAS and can perform well at the same conditions as the naturally selected strain. *rE. coli* appears best suited for general biomass conversion.

Short term, increasing xylose fermentation yield is the most direct route to commercial use of recombinant strains. In addition, for corn fiber, improving the arabinose yield is significant. Together they represent more than 90% of the potential gain. Achieving effective conversion of other hexose and oligomer sugars represents a much smaller opportunity.

Longer term, the many cost benefits of higher temperature operation can best be achieved with a new organism able to thrive at 50°C or higher. These include lower capital investment and lower operating cost. Consolidated bioprocessing: combining the enzyme production, saccharification and hydrolysis steps also may significantly lower capital and operating cost.

For strain development to proceed with confidence, representative process streams must be used. The effort requires an integrated, multi-disciplinary approach that includes chemical process design and operation coupled with molecular biology, biochemistry and genetics.

12. RECOMMENDATIONS

There are three key activities recommended to move the ethanologen development forward:

Support R&D Efforts That Fit Industry Needs.

Consistent with the purpose of this study, use the direction provided by the participants as a guide for supporting additional research and development efforts that match their requirements. Communicate these results to the researchers in this area, and solicit their expertise in accomplishing the desired results.

Provide Biomass Process Streams for Developers.

Presently, selection of the 'right' biomass and hydrolyzate process streams is important to insure limited resources are best deployed. Ag residues, especially corn stover, and corn fiber are likely initial choices for feedstock due to the availability, cost and infrastructure support. Bagasse is important worldwide, and in Louisiana for BC International. Hardwood is more distant from existing ethanol producers, with a higher cost. Coniferous, softwood is higher in lignin and not viewed as compatible for processing with the Ag residues.

These biomass choices should be evaluated and a judicious selection made for the substrates most likely to be commercialized.

Formalize Industry Dialog for Ethanologens.

A bi-annual meeting is suggested for the ethanologen topic specifically. It helps insure focus on the industrial needs and meeting their requirements.

A near continuous dialog is now maintained between NREL, DOE, industrial ethanol producers and others key for "Building the Bridge" to biomass produced ethanol. Many informal opportunities occur during symposia and meetings. Annual program reviews also are performed which look at the "big picture." A more detailed examination of the then current ethanologen situation is proposed every 2 years.

ACKNOWLEDGEMENT

We are grateful to the participants who gave generously of their time and shared their knowledge, to NREL for their support and encouragement, and to the US DOE Office of Energy Efficiency and Renewable Energy for funding the work.

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APPENDIX A

Study Participants

Charles Abbas
ADM Fermentation

Rod Bothast
NCAUR-USDA

Marion Bradford
A. E. Staley

David Cameron
Tembec Chemical Products

Ting Carlson
Cargill

Duane Kristenson
Chief Ethanol

Bill Farone, advisor to
Arkenol

Robert E. Lumpkin
SWAN

Steve Ramsden
Grain Processing Corporation

Larry Russo
Masada Corporation

Steve Schnrurrer
Minnesota Corn Processors

Bob Sutthoff
Enzyme Development Corporation

Pam Tetarenko
Vogelbusch USA

Jeff Tolan
Iogen

Gary Welsh
Williams Energy

Robyn Millichip Wells formerly with
Biocom International

Jennifer White/Kyd Brenner
Corn Refiners Association

Charles Wyman
BC International

Observers and Facilitators from NREL and DOE:

NREL: Mark Finkelstein, David Glassner, Mike Himmel, Min Zhang

DOE: John Ferrell, Marie Garcia, Gerson Santos-Leon

APPENDIX B

BIOMASS COMPOSITION STUDIES

The cellulose and hemicellulose composition of biomass from multiple sources is summarized in Table I. Values are Weight % on a dry basis.

TABLE I
Composition of Biomass Types

	Cellulose	Hemicellulose Composition			
Type	Glucan	Galactan	Xylan	Arabinan	Mannan
Corn Stover[1]	39-41	1.0-1.2	20-23	2.4-3.4	0.5-0.7
Corn Stover[2]	39	NR	20	2.0	NR
Corn Stover[3]	41	1.0	21	1.8	ND
Corn Stover[4]	36	NR	19	2.9	0.3
Switchgrass[1]	36-41	1.0-1.1	23-25	3.0-3.4	0.1-0.8
Serica Lespedez[1]	34-42	1.6-1.8	10-14	1.3-1.6	2.0-2.5
Short Rotation Hardwood[1]	39-44	0.7-1.2	13-17	0.4-1.1	1.5-1.3
Short Rotation Hardwood[3]	44-46	ND	16-17	0.4-0.7	0.9-1.2
Corn Fiber[5]	18	3.8	25	18	NR
Corn Fiber[6]	12	40			

NR = Reported, ND = Non Detected,

The study by Johnson et. al. (1995) examined changes in feedstock composition over a 26 to 52 week period. Overall structural losses were quite small. The range is shown in Table 1 for corn stover, switchgrass, sericea lespedeza and four varieties of hardwood.

The other studies included sample analysis as a related part of the paper. The change in composition in storage or by other factors was not addressed.

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APPENDIX C.

Zymomonas Use In the US Ethanol Industry, 1987-1989.

Biocom USA was established in 1987 to commercialize a proprietary, naturally selected Zymomonas strain, to replace yeast for the conversion of glucose to ethanol. The work was part of a thesis by Robyn Millichip, a student working towards a Masters degree at University of Queensland, Queensland, Australia, under the direction of Dr. Horst Doelle. Five papers were published and two patents were obtained. The references are listed below.

Pilot trials, 5 to 10 liters, were performed at ADM, Clinton Iowa and Staley, Loudon, TN. Plant scale trials followed at New Energy, Portales, NM; Shreveport Ethanol, Shreveport, LA and American Energy Fuels, Lincoln, NE. After some initial problems with seed inoculum scale up, long term, multi-month performance was demonstrated in the production fermentors up to 150,000 gallons.

No trials were made on any hydrolyzates of hemicellulose.

Typical fermentation conditions were 100° F, 12% to 15% ethanol and 3.6 to 3.8 pH. Conditions were anything but aseptic. The results were favorable. Robin completed her work and returned to Australia to receive her degree. At least one licensing offer was made. Terms could not be agreed on. Biocom decided to abandon the US market.

W. J. Wells, formerly with Biocom and American Eagle Fuels, received written FDA approval for the Zymomonas to be used in the animal feed. Copies are attached, Appendix C2. The CFR21 (1992 edition) did not indicate the Zymomonas approval. The 1992 edition of the AAFCO handbook also does not mention approval of Zymomonas.

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APPENDIX C.2.

FDA Approval as Food Additive

Initial Approval Letter

AUG 13 1987

Food and Drug Administration
Rockville MD 20857

DAF# 6009

William J. Wells
Vice President
Biocom International Limited
1954 Airport Road
Suite 251
Atlanta, GA 30341

Dear Mr. Wells:

We refer to your letters dated March 20, 1987; March 31, 1987; June 1, 1987; and July 29, 1987 seeking permission for the use of dried distillers grains from alcohol fermentation of small grains by Zymomas mobilis (a bacterial culture) as a feed ingredient. We understand that this bacterial culture is derived from "natural sources" and has not been chemically or genetically manipulated.

We have completed our review and have found your dried distillers grains product as an acceptable animal feed ingredient. However, should new information become available questioning its safety, we may withdraw this opinion. We wish for you to submit the results of Dr. Terry Klopfenstein's current research with feeding the DDG to cattle as progress reports become available. We consider distillers by-products to be a source of protein, fat and fiber and should not be labeled with claims other than this.

Specific comments follow:

1. This opinion only applies to the use of the naturally occurring bacteria Zymomas mobilis, not a chemically or genetically altered bacteria.

2. We will not object to the marketing of dried distillers grains, or condensed solubles or the combination.

3. You may wish to work with the Association of American Feed Control Officials to amend their current definitions for distillers by-products to provide for the use of this bacterial culture. We also recommend that if the chemical composition of these products is different from that listed for distillers by-products in the United States-Canadian Tables of Feed Composition you should apply for a new International Feed Number.

If you have questions on the points raised in this letter please let us know. We can be reached by telephone at (304) 443-5362.

Sincerely yours,

George Graber, PH.D.
Director
Division of Animal Feeds
Center for Veterinary Medicine

APPENDIX C3

FDA Approval as Food Additive

Follow Up Approval Letter

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

MAR 15 1988

INAD 6122

William J. Wells
Vice President
BioCom International Limited
1954 Airport Road
Suite 251
Atlanta, Georgia 30341

Dear Mr. Wells:

We refer to your letter dated February 15, 1988 in which you submitted the final report of cattle research completed at the University of Nebraska. This 103-day study compared the feeding of dried distillers grains and condensed distillers solubles from bacterial and yeast sources as well as an urea and soybean meal control.

Thank you for your promptness in submitting the interim and final reports. We believe that these reports support our original opinion that dried distillers grains and condensed distillers solubles from bacterial (Zymomas mobilis) fermentation of small grains is a safe feedstuff. As you know, information used to support a decision on the safe use of a food additive is available under our Freedom of Information (FOI) rules. Thus, if requested, we will release this final report to the public.

If you have questions regarding this letter, please let us know. We can be reached by telephone at (304) 443-9362.

Sincerely yours,

George Graber, Ph. D.
Director
Division of Animal Feeds
Center for Veterinary Medicine

APPENDIX D
Temperature and Pressure Effect on Alcohol Recovery and Inhibition

Alcohol Concentration and Temperature

Unlike a dissolved gas, the "solubility" of ethanol in the broth is not affected with increasing temperatures. Alcohol and water are completely miscible in the broth, mixing with each other in all proportions. A 12% alcohol solution remains 12% w/w as the temperature is increased. . . and is usually maintained at the desired level by adjusting the sugar feed rate for a fed batch fermentation system.

When the broth temperature is changed the composition of the vapor does change and is described by Raoult's Law:

$$P_{EtOH} = X_{EtOH} P_{EtOH}^{\circ} \qquad \text{Eq 1.}$$

$$P_{H_2O} = X_{H_2O} P_{H_2O}^{\circ} \qquad \text{Eq 2.}$$

Taking water and ethanol as the primary components, the pressure of ethanol is determined by the mole fraction, X_{EtOH}, times the vapor pressure of the pure component at that temperature, P_{EtOH}°, Eq 1. Water vapor pressure is given by Eq 2. so as temperature is adjusted, the vapor composition is changed slightly, and Lynd[1] has shown the effect on distillation cost is relatively small when the temperature is increased.

Inhibition, Alcohol Concentration and Pressure

Cockrem[2] ran several simulations to determine the relative impact of alcohol concentration, and operating pressure. At 65°C and 14.68 psia with a 12% w/w ethanol, the overhead vapor is about 55% ethanol on a CO_2 free basis.

When the pressure is lowered to 9.78 psia, the broth is 10.3% w/w ethanol and the overhead vapor is 52.5% ethanol. Dropping the pressure to 7 psia lowers the broth to only 7.9% w/w and the vapors are 46.6% ethanol.

A reduced pressure system can reduce alcohol inhibition, perhaps significantly, whilde still providing a concentrated overhead. There is virtually the same cost recovering 55%, 52.5% or 46.6% ethanol bin the vapor phase.

The author concludes an "economic optimum microorganism" exists in a space related to temperature, pressure, concentrations and inhibition effect.

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